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Abstract

1. Phenotypic variation is strongly impacted by environmental conditions experienced during development. Substantial laboratory research has shown that reptiles with flexible-shelled eggs are particularly sensitive to hydric conditions, yet research on nests in the wild is sparse.
2. In this 2-year field experiment, we explore the influence of hydric conditions during incubation on phenotypic traits of hatchling painted turtles (*Chrysemys picta*). Using a split-clutch design, we created two artificial nests adjacent to each maternally selected nest site. Half the eggs incubated in a nest that received regular supplemental watering, while the control nest was exposed to natural precipitation only.
3. Our results suggest that the influence of the hydric environment on developing reptilian embryos is context dependent. Supplemental water applied to nests in a drier than normal season elicited the expected biotic responses, based on laboratory experiments. However, when the soil surrounding *C. picta* eggs was already highly moist, the additional water from supplemental application effectively stunted embryonic development.
4. Our experiment confirms that hydric conditions of the soil during incubation in the wild can substantially influence phenotypic variation of reptiles with flexible-shelled eggs. Additionally, our experiment highlights the importance of complex interactions in the field that are often unexplored in laboratory experiments, reiterating the importance of validating laboratory work with field experiments.

Keywords

egg incubation, hydric conditions, phenotypic plasticity, reptile, soil moisture

Disciplines

Ecology and Evolutionary Biology | Other Ecology and Evolutionary Biology | Population Biology | Terrestrial and Aquatic Ecology

Comments

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Hydric conditions during incubation influence phenotypes of neonatal reptiles in the field

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Summary

1. Phenotypic variation is strongly impacted by environmental conditions experienced during development. Substantial laboratory research has shown that reptiles with flexible-shelled eggs are particularly sensitive to hydric conditions, yet research on nests in the wild is sparse.

2. In this 2-year field experiment, we explore the influence of hydric conditions during incubation on phenotypic traits of hatchling painted turtles (*Chrysemys picta*). Using a split-clutch design, we created two artificial nests adjacent to each maternally selected nest site. Half the eggs incubated in a nest that received regular supplemental watering, while the control nest was exposed to natural precipitation only.

3. Our results suggest that the influence of the hydric environment on developing reptilian embryos is context dependent. Supplemental water applied to nests in a drier than normal season elicited the expected biotic responses, based on laboratory experiments. However, when the soil surrounding *C. picta* eggs was already highly moist, the additional water from supplemental application effectively stunted embryonic development.

4. Our experiment confirms that hydric conditions of the soil during incubation in the wild can substantially influence phenotypic variation of reptiles with flexible-shelled eggs. Additionally, our experiment highlights the importance of complex interactions in the field that are often unexplored in laboratory experiments, reiterating the importance of validating laboratory work with field experiments.

Key-words: egg incubation, hydric conditions, phenotypic plasticity, reptile, soil moisture

Introduction

Realistic climate models project diverse environmental changes with high confidence in the coming decades (IPCC 2014). Among the most probable expectations are continued rises in global temperature and increased frequencies of extreme precipitation events (i.e. droughts and floods) (Rahmstorf & Coumou 2011). The biotic effects of such altered thermal environments are increasingly well understood theoretically, observationally and experimentally (Settele *et al.* 2014). However, biologists have spent considerably less effort exploring the consequences of radically modified hydric environments (e.g. Jergenson *et al.* 2014), especially for early life stages of freshwater animals in the wild, yet these impacts may be no less fundamental than those affected directly by changing temperatures.

Embryos are highly sensitive to environmental conditions experienced during development. Many factors, including

temperature, gas exchange, hormones, nutrition and hydric conditions, can exert major influences on phenotypic variation and survival across taxa (Lindstrom 1999; Phillips 2007; Wang *et al.* 2007; Fabrega *et al.* 2011). For early vertebrates in the Carboniferous, embryonic desiccation was a major barrier to a fully terrestrial life cycle. The evolution of the amniotic egg was an essential adaptation to overcome this barrier, because it allowed embryos to develop in an aqueous microenvironment, thereby minimizing desiccation in the drier landscape (Romer 1957, 1967; Little 1983; Sander 2012). Since then, amniotes have diversified tremendously, as have variations of the amniotic egg.

Some amniotes have further reduced the influence of external hydric conditions on the embryonic environment by evolving viviparity (e.g. mammals, some squamates) or a highly calcareous eggshell that reduces water exchange (e.g. birds, crocodilians, some geckos and turtles). Still, the eggs of many reptile species contain a minimal calcareous layer (Packard, Packard & Boardman 1982), rendering

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these flexible- and parchment-shelled eggs more vulnerable to hydric conditions during incubation than eggs with more rigid shells. For example, the parchment-shelled eggs of many lizards change mass while incubating in the laboratory, even doubling or tripling in moist environments (Tracy 1980; Andrews & Sexton 1981; Ackerman *et al.* 1985).

Nearly all experimental work on the effects of hydric conditions on embryonic reptiles has been performed in a laboratory setting. These studies find that hydric conditions affect numerous embryonic and offspring traits (Gutzke & Packard 1986; Packard *et al.* 1987; Packard 1999; Booth 2002). For example, turtle embryos in flexible-shelled eggs that are incubated in wetter environments consume their yolks faster, have seemingly higher rates of metabolism, have more rapid growth, grow larger and take longer to hatch than eggs incubated in drier environments (Packard & Packard 1986, 2000, 2001; Packard *et al.* 1987; Janzen *et al.* 1990; Miller & Packard 1992; Packard 1999). Laboratory studies of many squamate species reveal similar patterns in response to the hydric environments experienced during incubation (Packard, Packard & Boardman 1982; Phillips *et al.* 1990; Brown & Shine 2006; Reedy, Zaragoza & Warner 2013).

Whether such hydric effects are manifested in natural nests is unclear. This is partially due to the intensive effort required to measure soil water potential in subterranean nests (Packard, Miller & Packard 1992). Where measured, these data suggest that availability of water in nests is not constant (Packard, Packard & Gutzke 1985) and therefore these conditions can be difficult to mimic in a laboratory setting. Indeed, some observational studies of turtle nests have shown that the magnitude and pattern of egg mass change, presumably in response to soil moisture availability, can differ in laboratory and field incubation settings (Ratterman & Ackerman 1989). These patterns have been elegantly explored in cross-fostering and split-clutch manipulation experiments that decouple genetic/maternal effects and environmental effects in the field. One such experiment quantified soil water potential in nests, finding that moister nests induced longer incubation, yielded higher hatching success and produced larger hatchlings than drier nests (Cagle *et al.* 1993). Generally, however, these cross-fostering experiments have not quantified soil moisture, but have suggested that unmeasured hydric conditions are important nonetheless in influencing hatchling phenotypes (Shine, Elphick & Harlow 1997; Packard & Packard 2000, 2001; Booth, Feeney & Shibata 2013; Mitchell, Warner & Janzen 2013).

We supplement these efforts by utilizing a novel design that experimentally modified the hydric conditions of painted turtle (*Chrysemys picta*, Fig. 1) nests in two consecutive field seasons. To do this, we split clutches into two artificial nests adjacent to the maternally selected nest: the control nest in each pair experienced natural hydric conditions and the other nest in the pair received supplementary water to mimic a year with heavy precipitation.

From this experimental design, we assessed the effects of supplemental water on both the hydric and thermal environment of incubating nests and observed the consequences of this treatment on embryonic development and offspring phenotypic variation.

Materials and methods

STUDY SPECIES

The painted turtle (*Chrysemys picta*) is a freshwater species with a range that extends from Mexico to Canada (Starkey *et al.* 2003; Ernst & Lovich 2009). Painted turtles deposit flexible-shelled eggs in shallow subterranean nests from late spring to early summer. The calcareous layer is minimized in these eggs, facilitating egg expansion and permitting high eggshell water conductance (Ratterman & Ackerman 1989; Thompson & Speake 2004). The direction and magnitude of water movement between eggs and environment are likely passive (at least early in development; Warner, Moody & Telemeco 2011), depending on the relative water potentials of the eggs and soil. *Chrysemys picta* also exhibits temperature-dependent sex determination, such that high incubation temperatures produce females and low incubation temperatures produce males (Morjan 2003a).

The population used in this field experiment has been the focus of long-term research that addresses diverse facets of the reproductive ecology of *C. picta* (e.g. Janzen 1994; Schwanz *et al.* 2010; Warner, Jorgensen & Janzen 2010). This population resides in the backwaters of the Mississippi River and nests in the Thomson Causeway Recreation Area (TCRA), Thomson, Carroll County, Illinois, USA (41°57'15.80"N, 90°06'59.54"W). The preferred nesting areas at the TCRA contain predominantly short, maintained grass and variable canopy cover (Mitchell, Maciel & Janzen 2014). Soil types range from loamy to fine sandy loam, interspersed occasionally with areas of gravelly soils (Schwanz *et al.* 2009; <http://websoilsurvey.sc.egov.usda.gov/App/WebSoilSurvey.aspx>).

FIELD METHODS

Nesting turtles were located in May and June of 2012 and 2013. A total of 30 nests were excavated within *c.* 12 h of oviposition ($n = 13$ in 2012, $n = 17$ in 2013). Eggs were placed in plastic containers filled with soil from the nest cavity and were temporarily stored in Styrofoam coolers. Eggs from each clutch were weighed to the nearest 0.01 g, and labelled and randomly assigned to one of the two treatments (control nests and watered nests). Two artificial nests were constructed, each 50 cm from the natural nest on opposite sides and at the same elevation and containing one-half the original clutch size. To ensure consistency and environmental relevance, B.L.B. constructed all experimental nests in a flask shape with a trowel and spoon to mimic the maternal nest (depth of 7 cm; Morjan 2003b). A Thermocron iButton, programmed to record temperature hourly, was wrapped in latex and Parafilm and placed among the eggs in the centre of the nest cavity for three pairs of nests in 2012 and for 15 pairs of nests in 2013. After eggs were placed in a cavity, the nest was backfilled with soil, protected from predation with 1 cm mesh wire hardware cloth (25 cm × 25 cm), and mapped for relocation.

Control nests received water from natural precipitation events in June and July (5.13 cm in 2012 and 18.76 cm in 2013), but were otherwise unaltered. Watered nests received the equivalent of an additional 6.0 cm of rainfall per week, split into two watering events (3.0 cm of rainfall, twice per week). To simulate each rainfall event, we slowly sprinkled 2.3 L of water from a watering can over a 30.8 × 24.8 cm area, delimited by a bottomless plastic container. Our treatment values mimicked the wettest June and July

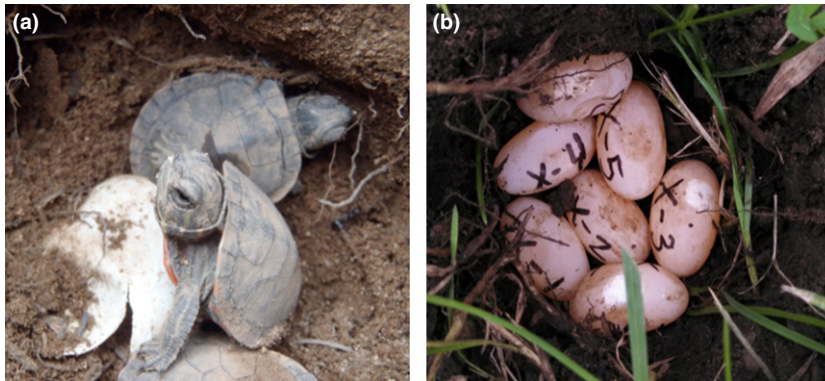


Fig. 1. (a) Hatchling painted turtle emerging from nest. (b) Experimental clutch of painted turtle eggs from this experiment.

recorded from 1939 to 2011. The weather station located 5.5 km south of the TCRA recorded 27.25 cm of rain in June 1993 and 20.85 cm of rain in July 1963, totalling 48.1 cm of rain over the 2 months.

The percentage volumetric water content (VWC%) for all nests was measured at each watering event by a Hydrosense instrument (CD620, Campbell Scientific, Australia). The two metal probes of the instrument were inserted to a depth of 6 cm. Four measurements were taken (one from each cardinal direction from the nest) and were averaged. The VWC% for each watered nest was obtained twice, immediately before a watering event and again within *c.* 5 min after a watering event. The VWC% for each control nest was also measured at this time. VWC% readings preceding a watering event represent the minimal difference between the paired nests and such readings after a watering event represent the maximal difference.

Nests were excavated on 21 July 2012 and 1 August 2013. The nearly hatched eggs were reweighed immediately in the field and then stored in Styrofoam containers for transport to Iowa State University. In the laboratory, the clutches were placed in plastic containers with moist vermiculite (-150 kPa) in incubators at constant 28.5°C . Eggs were checked daily for pipping (egg shell severed). Upon pipping, a bottomless cup was placed around the egg to insure accurate identification of the hatchling. Within 24 h of hatching, a turtle was weighed, measured [carapace length (CL), carapace width (CW), plastron length (PL)] and then sacrificed via injection of 0.5 mL of 1 : 1 deionized water : Sleepaway near the heart. The hatchling was subsequently dissected to assess gonadal sex and to remove the residual yolk. The carcass and the yolk were weighed separately and then placed in a drying oven at 40°C until reaching a constant mass. Final dry masses were recorded, and the carcass and yolk were discarded.

STATISTICAL METHODS

Using the MIXED procedure in SAS, we performed analysis of variance (ANOVA) and covariance (ANCOVA) to compare abiotic properties and turtle phenotypes between control and watered nests. In all analyses, clutch was modelled as a random factor and treatment was modelled as a fixed factor. In these analyses, clutch variance comprises factors such as genetics and maternal effects (i.e. nest-site choice/oviposition date/egg size where not included as a covariate). From iButtons, we extracted overall daily mean, mean daily minimum, mean daily maximum and mean daily range of nest temperatures and used these values as response variables. For additional abiotic response variables, we similarly calculated average VWC% readings for each control and watered nest both before and after watering events. Phenotypes that served as response variables included change in egg mass during incubation, duration of incubation, carapace length, carapace width, plastron length, body mass, wet yolk mass, dry yolk mass, wet carcass mass

and dry carcass mass. Covariates (initial egg mass, days spent in field, etc.) were included in models where appropriate. Eggs that did not hatch were excluded from phenotypic analyses. Survival (proportion of eggs successfully hatching from a nest) and sex ratio (proportion male) were analysed with a generalized linear mixed model (PROC GLIMMIX) with a binomial distribution and a logit link function. Again, clutch was a random factor and treatment was a fixed factor.

Results

Summer 2012 was one of the hottest and driest on record at our field site (Schwanz *et al.* 2010; Warner, Jorgensen & Janzen 2010). During the field incubation period in 2012, the average ambient temperature was $24\text{--}37^{\circ}\text{C}$ and 5.13 cm of rain fell (data obtained from Clinton, IA, USA; National Climatic Data Center (www.ncdc.noaa.gov)). Summer 2013 was thermally typical and characterized by a fairly wet May through mid-June, yet had well below average precipitation from mid-June through the remainder of the incubation period (Illinois State Water Survey (www.sws.uiuc.edu)). During the field incubation period in 2013, the average ambient temperature was $22\text{--}65^{\circ}\text{C}$ with 18.76 cm of precipitation (data obtained from Clinton, IA, USA; National Climatic Data Center (www.ncdc.noaa.gov)). In 2012, we had 26 experimental nests from 13 clutches. These 13 clutches contained 113 eggs, 62 of which hatched successfully. In 2013, we had VWC% data from all 34 experimental nests from 17 clutches. Temperature logger malfunctions reduced the thermal data set to 15 clutches. Four clutches produced no successful hatchlings (2 clutches flooded, 2 were infertile). Consequently, the data set for survival and phenotypic analyses included 13 clutches, which contained 136 eggs, 93 of which hatched successfully.

In both years, watered nests had higher VWC% compared to control nests, especially immediately after a watering event, where the difference was roughly twofold (Table 1). Overall, nests had 5–9% lower VWC in 2012 than in 2013. In both years, watered nests also experienced cooler thermal environments compared to control nests, although these differences were most substantial for mean and maximum temperatures in 2012 (Table 2). Overall, 2012 nests were warmer than 2013 nests, particularly in thermal maxima and range ($2\text{--}4^{\circ}\text{C}$ difference).

Table 1. Comparison of volumetric water content percentage (VWC%) between control nests and watered nests of *Chrysemys picta* prior to a watering event (before) and immediately after a watering event (after) in 2012 and 2013. Least squares mean \pm one standard error is reported. Bold text represents statistically significant results.

Year	VWC%	LSM \pm SE	Treatment statistic
2012	Control	11 \pm 1	–
2012	Watered Nest Before	12 \pm 1	$F_{1,12} = 1.75, P = 0.211$
2012	Watered Nest After	24 \pm 2	$F_{1,12} = 105.72, P < \mathbf{0.001}$
2013	Control	17 \pm 3	–
2013	Watered Nest Before	18 \pm 3	$F_{1,16} = 3.62, P = 0.075$
2013	Watered Nest After	32 \pm 3	$F_{1,16} = 272.46, P < \mathbf{0.001}$

The proportion of eggs successfully hatching was similar in watered and control nests in 2012 (watered = 0.56 ± 0.37 , control = 0.52 ± 0.35 , $F_{1,12} = 0.04$, $P = 0.84$). Eggs from watered nests had larger, positive changes in mass and averaged a day longer to hatch compared to eggs in control nests (Table 3). Accordingly, hatchlings from eggs in watered nests were larger than hatchlings from control nests, but significantly so only for carapace width (Table 3). At the same time, hatchlings from watered nests had smaller yolk masses and marginally larger carcass masses than hatchlings from control nests (Table 3). Overall, 11% of hatchlings were male, all of which were produced from the same clutch (both experimental nests from that clutch had mixed sex ratios); thus, we did not statistically analyse sex ratio in 2012. For context, hatchlings from natural nests in a nearby observational study were 18% male in 2012 (F. J. Janzen, unpublished data).

In 2013, watered nests had nearly 25% lower survival than control nests, though this difference was not statistically significant (watered = 0.54 ± 0.34 , control = 0.82 ± 0.26 , $F_{1,12} = 2.24$, $P = 0.16$). Eggs from watered nests had larger, positive changes in mass and averaged 3 days longer to hatch compared to eggs in control nests (Table 3), which was the same pattern observed in 2012. Again, watered nests had varied effects on hatchling phenotype, but the patterns were reversed compared to 2012. In brief, hatchlings from eggs in the watered nests were smaller in essentially all

respects than hatchlings from eggs in the control nests (Table 3). Sex ratios (proportion male) were similar in the watered and control nests (watered = 0.39 ± 0.49 , control = 0.44 ± 0.43 , $F_{1,11} = 0.14$, $P = 0.71$). Hatchlings from natural nests in 2013 were 60% male (F. J. Janzen, unpublished data).

Discussion

Numerous laboratory experiments have established that hydric conditions during embryogenesis elicit various biotic responses in reptiles, especially in those with flexible-shelled eggs (reviewed in Ackerman 1991; Packard & DeMarco 1991; Belinsky *et al.* 2004). Moreover, several field studies have identified correlations between measures of nest hydric environments and offspring traits consistent with findings from those laboratory experiments (e.g. Cagle *et al.* 1993). Even so, our 2-year experiment is the first to manipulate the hydric environment in the field and quantify its effects on phenotypic variation in reptile offspring.

Our results suggest that the influence of the hydric environment on developing reptilian embryos is context dependent. That is, supplemental water applied to nests in a drier than normal season elicited the expected biotic responses, based on laboratory experiments. However, when the soil surrounding *C. picta* eggs was already highly moist, the additional water from supplemental application effectively stunted embryonic development. The majority of laboratory work has typically exposed eggs to constant hydric conditions within a range that is generally suitable for embryonic development. Yet, natural nests (and nests from our experiment) are exposed to a wide range of constantly changing conditions, including those that could desiccate or drown eggs, which has not been well explored in the laboratory. Our results generally accord with prior laboratory work, but also provide new insights and highlight areas in which laboratory work has failed to mimic the complexities of nature.

TREATMENT EFFECTS ON NEST ENVIRONMENT AND OFFSPRING PHENOTYPES

Water potential is the typical measure used to predict the movement of water and is traditionally applied to understand

Table 2. Comparison of thermal properties between control and watered experimental *Chrysemys picta* nests in 2012 and 2013. Bold text represents statistically significant results.

Year	Temperature (°C)	Control nest LSM \pm SE	Watered nest LSM \pm SE	Treatment statistic
2012	Mean	25.4 \pm 0.9	24.5 \pm 0.9	$F_{1,2} = 27.97 P = \mathbf{0.034}$
2012	Minimum	20.8 \pm 0.6	20.6 \pm 0.6	$F_{1,2} = 3.48 P = 0.203$
2012	Maximum	32.2 \pm 1.3	30.1 \pm 1.3	$F_{1,2} = 17.14 P = \mathbf{0.054}$
2012	Range	11.4 \pm 0.8	9.6 \pm 0.8	$F_{1,2} = 12.10 P = 0.074$
2013	Mean	24.0 \pm 0.3	23.9 \pm 0.3	$F_{1,14} = 1.03 P = 0.327$
2013	Minimum	21.1 \pm 0.2	20.9 \pm 0.2	$F_{1,14} = 2.54 P = 0.133$
2013	Maximum	28.4 \pm 0.5	28.1 \pm 0.5	$F_{1,14} = 0.55 P = 0.473$
2013	Range	7.3 \pm 0.4	7.2 \pm 0.4	$F_{1,14} = 0.10 P = 0.759$

Table 3. Comparison of hatchling traits between control and watered *Chrysemys picta* nests in 2012 and 2013. Covariate for hatchling linear measurements and hatchling mass is initial egg mass. Covariate for incubation duration was the number of days spent in the field. Bold text represents statistically significant results.

Year	Phenotype	Control nest LSM \pm SE	Watered nest LSM \pm SE	Clutch variance	Covariate statistic	Treatment statistic
2012	Initial Egg Mass (g)	7.03 \pm 0.26	6.99 \pm 0.26	0.91		$F_{1,48} = 0.20$ $P = 0.658$
2012	Egg Mass Change (g)	0.85 \pm 0.17	1.49 \pm 0.17	0.53	$F_{1,46} = 1.62$ $P = 0.210$	$F_{1,46} = 28.85$ $P < 0.001$
2012	Incubation Duration (d)	68 \pm 1	69 \pm 1	0.81	$F_{1,47} = 11.20$ $P = 0.016$	$F_{1,47} = 4.17$ $P = 0.046$
2012	Carapace Length (mm)	26.0 \pm 0.3	26.3 \pm 0.3	0.10	$F_{1,48} = 14.68$ $P < 0.001$	$F_{1,48} = 0.85$ $P = 0.360$
2012	Carapace Width (mm)	22.8 \pm 0.2	23.4 \pm 0.2	0.22	$F_{1,47} = 9.62$ $P = 0.003$	$F_{1,47} = 6.02$ $P = 0.018$
2012	Plastron Length (mm)	25.3 \pm 0.3	25.4 \pm 0.3	0.51	$F_{1,47} = 13.37$ $P = 0.001$	$F_{1,47} = 0.36$ $P = 0.553$
2012	Hatchling Mass (g)	5.18 \pm 0.08	5.27 \pm 0.09	0.38	$F_{1,47} = 63.00$ $P < 0.001$	$F_{1,47} = 1.52$ $P = 0.224$
2012	Wet Yolk Mass (g)	0.49 \pm 0.03	0.43 \pm 0.03	0.45	$F_{1,47} = 28.10$ $P < 0.001$	$F_{1,47} = 4.81$ $P = 0.033$
2012	Dry Yolk Mass (g)	0.26 \pm 0.02	0.22 \pm 0.02	0.46	$F_{1,47} = 22.58$ $P < 0.001$	$F_{1,47} = 4.79$ $P = 0.034$
2012	Wet Carcass Mass (g)	4.87 \pm 0.10	5.03 \pm 0.10	0.26	$F_{1,47} = 25.17$ $P < 0.001$	$F_{1,47} = 2.28$ $P = 0.138$
2012	Dry Carcass Mass (g)	1.06 \pm 0.03	1.10 \pm 0.03	0.57	$F_{1,47} = 12.97$ $P = 0.008$	$F_{1,47} = 3.96$ $P = 0.052$
2013	Initial Egg Mass (g)	6.95 \pm 0.24	6.96 \pm 0.24	0.93		$F_{1,79} = 0.10$ $P = 0.757$
2013	Egg Mass Change (g)	1.56 \pm 0.22	2.63 \pm 0.22	0.75	$F_{1,76} = 5.97$ $P = 0.017$	$F_{1,76} = 116.68$ $P < 0.001$
2013	Incubation Duration (d)	74 \pm 1	77 \pm 1	0.74	$F_{1,77} = 1.67$ $P = 0.200$	$F_{1,77} = 25.57$ $P < 0.001$
2013	Carapace Length (mm)	25.5 \pm 0.2	25.1 \pm 0.2	0.20	$F_{1,78} = 51.6$ $P < 0.001$	$F_{1,78} = 6.54$ $P = 0.013$
2013	Carapace Width (mm)	23.0 \pm 0.2	22.6 \pm 0.2	0.27	$F_{1,78} = 22.94$ $P < 0.001$	$F_{1,78} = 2.96$ $P = 0.089$
2013	Plastron Length (mm)	24.77 \pm 0.24	24.0 \pm 0.3	0.29	$F_{1,78} = 30.92$ $P < 0.001$	$F_{1,78} = 11.95$ $P = 0.001$
2013	Hatchling Mass (g)	4.88 \pm 0.07	4.71 \pm 0.08	0.47	$F_{1,78} = 58.49$ $P < 0.001$	$F_{1,78} = 10.32$ $P = 0.002$
2013	Wet Yolk Mass (g)	0.55 \pm 0.05	0.56 \pm 0.05	0.63	$F_{1,78} = 2.950$ $P = 0.090$	$F_{1,78} = 0.03$ $P = 0.865$
2013	Dry Yolk Mass (g)	0.33 \pm 0.03	0.34 \pm 0.03	0.63	$F_{1,78} = 2.580$ $P = 0.112$	$F_{1,78} = 0.19$ $P = 0.664$
2013	Wet Carcass Mass (g)	4.52 \pm 0.09	4.32 \pm 0.10	0.38	$F_{1,78} = 20.71$ $P < 0.001$	$F_{1,78} = 6.47$ $P = 0.013$
2013	Dry Carcass Mass (g)	0.98 \pm 0.03	0.96 \pm 0.03	0.52	$F_{1,78} = 14.56$ $P < 0.001$	$F_{1,78} = 1.99$ $P = 0.162$

the relation of water to reptile eggs. Measuring water potential in the field was logistically infeasible for our experiment, but VWC was readily measureable. Using a standard curve that relates VWC to water potential for various soil types (Saxton *et al.* 1986), we can approximate the range of water potentials likely experienced by our experimental nests (Table 1). These conversions allow us to better interpret our results in the context of prior work.

In 2012, the central United States experienced severe drought conditions. In our experiment, control nests averaged *c.* 11% VWC, which ranges from -450 to -1500 kPa for the soil types at our site. Soils at -1500 kPa tightly hold water, while water uptake from soil is more probably at higher water potentials. Eggs in control nests increased their mass by 12% on average during incubation. Our watered nests ranged from 0 to -20 kPa (fairly wet) immediately after watering, but dried nearly to the same point as the control nests prior to the next watering event. Watered nests were also slightly cooler than control nests. Eggs from these watered nests gained 79% more water (21.5% egg mass increase) and took marginally longer to hatch, than eggs from control nests. Hatching success was similar between treatments (*c.* 52–56%) and lower than in natural nests incubating at the field site that year (73%, F. J. Janzen, unpublished data). Of the surviving hatchlings, those from watered nests were generally larger than those from control nests, with less residual yolk, indicating these hatchlings were able to convert more yolk into tissue than their siblings in the drier, control nests. This suite of phenotypic patterns thus accords with prior laboratory experiments (Packard & Packard 1986, 2000, 2001;

Packard *et al.* 1987; Janzen *et al.* 1990; Miller & Packard 1992; Packard 1999).

In 2013, a year with more typical weather conditions, average water potentials of control nests ranged from -20 kPa to -175 kPa. At this water potential, egg mass increased substantially (22.5% egg mass gain) during incubation. Water potential of watered nests was *c.* 0 kPa for all soil types after watering to about -10 to -150 kPa just before watering again. At 0 kPa, soil is saturated with water, and nests were likely experiencing hypoxic or anoxic conditions (Packard & Packard 1988; Booth 1998). Watered nests were slightly cooler than control nests, with eggs gaining 72% more mass (38% mass increase) and taking somewhat longer to hatch than eggs from control nests. This general pattern is congruent with the results from 2012 and prior laboratory work. Unexpectedly, however, hatchlings from the watered nests were generally smaller than those from control nests. Additionally, eggs from watered nests had considerably lower hatching success (watered = 54%, control = 82%; nearby natural nests = 70% (F. J. Janzen, unpublished data)). This pattern may seem counterintuitive, as most laboratory studies have suggested that wetter substrates produce larger hatchlings and yield higher hatching success (e.g. Packard 1999). Kam (1993), however, showed that hypoxic conditions during incubation can slow embryonic growth and metabolism and reduce hatchling mass in a turtle with flexible-shelled eggs. Similarly, Kam (1994) simulated flooding conditions and found that submersion in water can drastically reduce hatching success in such species. Thus, a likely explanation for this surprising pattern is that water

saturation induced anoxic or hypoxic conditions, which disrupted embryonic development in eggs from watered nests.

While our supplemental water treatment mimicked extremely heavy rainfall, the nest conditions induced experimentally in both years are environmentally relevant. Large-scale flooding events occur occasionally at our field site during incubation (Janzen 1994; Jergenson *et al.* 2014). It is also common for subsets of nests in low-lying microhabitats to be submerged multiple times from small-scale flooding events after heavy rains (i.e. nest under a puddle, T. S. Mitchell, personal observation).

Soil moisture and soil thermal conductivity and diffusivity vary positively but nonlinearly (Al Nakshabandi & Kohnke 1965). Solar heat input at the soil surface is drawn deeper into the soil column when soil moisture is high, resulting in a cooler nest near the soil surface. Thus, as expected, supplemental watering cooled nests, although the magnitude of this effect differed by year. In the drier year (2012), increasing water content of the soil increased thermal conductivity greatly, resulting in a cooler nest. In 2013, supplemental water had a negligible influence on thermal conductivity, and consequently there was little influence on nest temperature.

GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

Our findings could have important ecological implications for fitness in reptiles with flexible or parchment-shelled eggs. Growth (Froese & Burghardt 1974) and survival (Swingland & Coe 1979) of hatchling turtles may be affected by the size they attain before hatching (Morris *et al.* 1983). Substantial evidence also suggests that relatively large neonates typically have higher survival during early life stages (Sinervo *et al.* 1992; Janzen, Tucker & Paukstis 2000a,b; Tucker 2000; Warner & Andrews 2002). In turtles, enhanced migration performance of larger hatchlings reduces exposure to predators under natural conditions after leaving the nest (Janzen, Tucker & Paukstis 2007), supporting the model that links phenotype, performance and fitness (Irschick 2003).

Our water manipulations had context-dependent effects on nest temperature as well as on offspring. This finding accords with general understanding of soil physics (Al Nakshabandi & Kohnke 1965), but emphasizes the biological relevance for subterranean nesting animals. For example, nests near the soil surface may be warmer during dry years than wet years, even if air temperature and solar radiation are equivalent. Additionally, the influence of rainfall events on nest temperature will depend on the standing soil moisture. This interaction between soil temperature and soil moisture will be important when predicting the biological effects of climate change on organisms with subterranean nests, particularly those like *C. picta* with temperature-dependent sex determination. In most climate models, precipitation patterns are expected to change drastically, increasing the risk of drought and flooding events (Rahmstorf & Coumou 2011; IPCC 2014). This

interaction could have management implications as well; for example, cooling sea turtle nests with irrigation has been tested as a possible method to mitigate effects of a warming climate (Jourdan & Fuentes 2013).

Our experiment has generated outstanding questions that could be addressed with future research. Quantifying relationships between water content, water potential, soil temperature and hatchling phenotypes in a variety of soil substrates would be particularly insightful. Additionally, substantial research has investigated diverse factors influencing maternal nest-site choice, and for many organisms, hydric conditions are a primary factor. Field research building off laboratory work to investigate the importance of moisture in nest-site choice is warranted (Plummer & Snell 1988; Delaney *et al.* 2013; Reedy, Zaragoza & Warner 2013). Finally, further laboratory research is needed that better mimics the complexity of nature, by inducing fluctuating thermal and hydric conditions (e.g. Delmas *et al.* 2008; Les, Paitz & Bowden 2009; Warner & Shine 2011) within environmentally relevant bounds.

This foray into experimentally quantifying the effects of hydric conditions on phenotypes in the field has yielded novel insights. Laboratory experiments provided an excellent framework in which to ground our hypotheses and were useful in predicting plastic responses under some circumstances. Yet, we induced ecologically relevant environmental conditions that produced unexpected results as well, and we provide suggestions for follow-up laboratory work. It is important to experimentally validate the ecological relevance of laboratory conditions before extrapolating those results for inference in the field (St Juliana & Janzen 2007; Warner & Shine 2009). Additionally, our results emphasize fundamental interactions between the hydric and thermal environment in soils, a relationship that will be increasingly important to accommodate in models and experiments to accurately predict responses to climate change (Settele *et al.* 2014). This experiment highlights the complexities of predicting phenotypically plastic responses in nature from studies in the laboratory that employ relatively simplistic environmental conditions.

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Data accessibility

Data are deposited in the Dryad Digital Repository: doi:10.5061/dryad.bj55p (Bodensteiner *et al.* 2014).

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